MHC class II antigen presentation by B cells in health and disease

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Chapter 2

BCR-mediated internalization of *Salmonella*: a novel pathway for autonomous B cell activation and antibody production

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Abstract

The present paradigm is that primary B cells are non-phagocytosing cells. In this study, we demonstrate that human primary B cells are able to internalize bacteria when the bacteria are recognized by the B cell receptor (BCR). BCR-mediated internalization of *Salmonella typhimurium* results in B cell differentiation and secretion of anti-*Salmonella* Ab by the *Salmonella*-specific B cells. In addition, BCR-mediated internalization leads to efficient Ag delivery to the MHC class II Ag-loading compartments, even though *Salmonella* remains vital intracellularly in primary B cells. Consequently, BCR-mediated bacterial uptake induces efficient $CD4^+$ T cell help, which boosts *Salmonella*-specific Ab production. BCR-mediated internalization of *Salmonella* by B cells is superior over extracellular Ag extraction to induce rapid and specific humoral immune responses and efficiently combat infection.

Introduction

Defense against pathogens is essential for survival and is controlled by the innate and acquired arms of the immune system. Ag presentation by B lymphocytes is needed to generate high-affinity Abs (1, 2). Development of an effective humoral immune response is mediated by two actions of the BCR: transmembrane signaling through BCR complexes to induce B cell differentiation and Ag internalization for processing followed by MHC class II-mediated presentation to acquire T cell help. The proper execution of both actions requires binding of a polyvalent Ag to multiple BCR molecules. Indeed, many B cell Ags are polyvalent as they are bound in multiple copies to the particulate surfaces of microbes or cells (3).

The role of $CD4^+$ T cells in the induction of protective immunity against pathogens is well established (4, 5). $CD4^+$ T cell activation requires MHC class II Ag presentation after Ag processing in the endocytic pathway and subsequent binding of antigenic peptides to MHC class II molecules, a process controlled by the MHC class II chaperones HLA-DM and HLA-DO (6-8). B cells use their BCR to concentrate specific Ag to the Ag-loading compartments (termed MIIC for MHC class II containing compartment) for loading of Ag onto newly synthesized MHC class II molecules (3). Besides internalization of Ag, the BCR drives intracellular targeting by accelerating the delivery of Ag to MIICs (9). Furthermore, BCR signaling ignited by Ag induces acidification of the MIICs, which favors Ag loading onto newly synthesized MHC class II molecules (10). Together, these cellular adaptations enable B cells to preferentially present specific Ags that have been internalized via the BCR to $CD4^+$ T cells.

Since primary B cells are considered to be not phagocytic, it is unclear how they acquire Ags from bacteria for Ag presentation. B cells can present particulate Ags in the context of MHC class II (11-14) and are able to extract Ag from a non-internalizable surface (15). Studies on MHC-mediated presentation of BCR-specific Ags are mainly performed with soluble Ags or with pre-cross-linked anti-BCR Abs. We used *Salmonella typhimurium* as a model system to study MHC class II Ag presentation of particulate, polyvalent Ags, and B cell activation. Being facultative intracellular pathogens, immunity to *Salmonella* requires adequate humoral and cell-mediated immune responses (16, 17). *Salmonella* invades host macrophages, but also many other cells and establishes an intracellular niche inside discrete vacuoles, known as *Salmonella*-containing vacuoles or SCV (18). This feature of *Salmonella* is considered crucial for their survival and pathogenicity (19, 20).

In this report we show that B cells are highly efficient phagocytes of inert particles, like beads, when these particles are recognized by the BCR. B cells are thus ligand-selective phagocytic cells. BCR-mediated internalization of *Salmonella* generates autonomous B cell activation and rapid anti-*Salmonella* Ab secretion. Immediate intimate contact and fusion occurs between MIICs and SCVs. Consequently, Ag presentation and proliferation of *Salmonella*-specific $CD4^+$ T cells is induced. Although BCR-mediated internalization suffices to drive Ab production, T cell help further improves the response. The observation that B cells can proliferate and differentiate autonomously after *Salmonella* uptake is important in light of the remaining Ab responses to pathogens when $CD4^+$ T cell help fails, as is the case in HIV patients.

Materials and methods

Antibodies, beads and fluorophores

Goat anti-mouse IgG-conjugated Dynabeads M-450 (Dynal Biotech) were coated with mAb anti-human IgM (MH15; Sanquin). The anti-human IgM antibody (MH15) was mixed with mAb anti-*S. typhimurium* LPS (1E6; Biodesign International) and rat anti-mouse IgG1 (RM161.1; Sanquin) to generate BCR-LPS tetrameric Ab complexes. The mAb anti-human HLA-DR (L243) (21) was used to block MHC class II-TCR interaction in T cell proliferation assays. For immunoelectron microscopy (EM), mAb anti-human CD63 (435; Sanquin), rabbit anti mouse (Nordic) and gold (10 nm)-conjugated protein-A (EM Laboratory, Utrecht University, The Netherlands) were used. $F(ab')_2$ fragments of MH15 were generated by standard pepsin digestion.

PE-conjugated anti-IgM was obtained from Sanquin (MH15-PE), anti-CD27-PE and IgG1-PE isotype control from BD Biosciences. Fluorescent secondary Ab goat anti-mouse Alexa Fluor 633 and Texas Red-phalloidin were obtained from Molecular Probes and 4',-diamidino-2-phenylindolyne (DAPI) from Sigma-Aldrich.

Transfectant cell lines

The pcDNA3 DOβGFP (22) and DR1βGFP (23) constructs have been described before. DOβGFP and DR1βGFP were demonstrated to form complexes with their respective endogenous α-chain. Transfections were performed by electroporation using a Gene Pulser II with Capacitance Extender (Bio-Rad). Stable transfectants of the human B cell line Ramos were selected and maintained in RPMI 1640

supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME in the presence of 2000 µg/ml G418 (Life Technologies). Stable expression of the GFP-tagged proteins was verified by Western blotting and ensured by regular selection of positive cells by FACS sorting.
NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) (24) were cultured in IMDM supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME and 500 µg/ml G418 (Life Technologies). 3T3-CD40L cells were harvested, irradiated with 30 Gy (Gammator M38-1, MDS Nordion) and seeded without antibiotics in 96 wells flat bottom plates ($2*10^4$ cells per well) to form a confluent monolayer overnight.

Bacterial Strains

S. typhimurium SL1344 (*Salmonella*) (25), GFP-*Salmonella* (26) and mRFP-*Salmonella* (27) were described before. GFP-*Salmonella* defective in SPI-1 (invA mutant) or SPI-2 (ssrA mutant) were a kind gift from M. Rescigno. Staphylococcus aureus expressing GFP (RN4220 with pWVW189GFP) was a kind gift from S.A.J. Zaat. Bacteria were grown in Luria-Bertani (LB) broth with antibiotics overnight at 37°C while shaking, subcultured at a dilution of 1/33 in fresh LB media, and incubated at 37°C while shaking for 3.5 hours. Bacteria were washed twice with PBS, incubated 1/25 with the BCR-LPS tetrameric Ab complexes for 30 min at room temperature, and washed twice to remove unbound Abs. Dead *Salmonellae* were bacteria fixed with paraformaldehyde (3.7% in PBS).

Lymphocyte isolation and proliferation assay

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS) from a buffycoat obtained from healthy donors after informed consent (Sanquin). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads and DETACHaBEAD (Dynal Biotech), according to the manufacturer's instructions.
B lymphocytes were incubated for 40 min at 37°C with *Salmonella* without antibiotics. Next, cells were washed four times and cultured for 1 h in medium containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-internalized bacteria. Cells were washed and cultured in RPMI 1640 medium without phenol red, supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME, 20 µg/ml human apo-transferrin (Sigma-Aldrich);

depleted for human IgG with protein G sepharose (Amersham Biosciences)), and 10 µg/ml gentamycin. One x 10^5 B cells and 5 x 10^4 T cells were cultured in 200 µl at 37°C in the presence of 5% CO_2 in 96 wells round bottom plates (Greiner Bio-One). The maximum proliferation capacity of T lymphocytes (varying between 35 to 60 x 10^3 cpm) was established by stimulation with anti-CD3 (CLB.T3/4.E, Sanquin) and anti-CD28 (CLB.CD28/1, Sanquin) which were both used at 1 µg/ml. After 5 and 12 days, 150 µl of supernatant was collected for Ab measurement and fresh medium was added. To study the kinetics of Ag presentation, B cells incubated with *Salmonellae* were irradiated with 60 Gy at indicated time points before incubation with T cells. For B/T cell proliferation assays, after 5 days of culturing [^{3}H]thymidine (GE Healthcare) was added at a final concentration of 1 µCi/ml (37 kBq/ml) for 16 h. Cells were harvested on glass fiber filters (Wallac) and radioactivity was measured with a 1205 Betaplate liquid scintillation counter (Wallac). For blocking experiments, B cells were preincubated with 5 µg/ml anti-HLA-DR (L243) for 30 minutes before T cells were added. For the Ag-specificity assay, $CD4^+$ T cells were CFSE labeled and cocultured with B cells that had taken up *Salmonellae*. The dividing T cells were sorted after 6 days and cultured with 10 IU/ml IL-2 (Chiron) for 6 more days. PBMCs were labeled with CFSE and incubated with tetanus toxoid (7,5 µg/ml; Statens Serum Institut, Copenhagen, Denmark) for 11 days, with 10 IU/ml IL-2 added on day 6, and proliferating $CD4^+$ T cells were sorted. B cells from the same donor were incubated with *Salmonellae* and PBMCs from the same donor with Tetanus Toxoid, irradiated after 18 h and then the sorted T cells were added for 2 days before [^{3}H]thymidine was added for 16 h.

FACS analyses

Freshly isolated primary B cells were incubated with *Salmonella*, washed four times, and cultured for 1 h in medium containing 100 µg/ml gentamycin. Cells were incubated with directly labeled Abs and for LPS staining cells were incubated with anti-LPS Ab and subsequently with Alexa Fluor 633-conjugated goat-anti-mouse Ab. Cells were washed with PBS containing 0.1% BSA. Lymphocytes were gated by forward and side scatter and dead cells were excluded by staining with DAPI. Two hundred thousand events were acquired on an LSR II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

FACS sorting of B cells that had internalized uncoated living bacteria was performed on a MoFlo Sorter (Dakocytomation), populations were >75% purified.

Live cell imaging and EM analyses

For CLSM analysis, coverslips were coated with 1 mg/ml Poly-L-lysine (Sigma-Aldrich) for 1 h and washed thoroughly with Aquadest and dried on air. Cells were allowed to attach on the coated coverslips for 15 min and subsequently beads or *Salmonellae* were added. For visualization of the actin cytoskeleton, cells were fixed with 3.7% paraformaldehyde and stained with Texas Red-phalloidin and DAPI. Confocal analysis was performed at 37°C using a Leica TCS SP confocal laser scanning microscope equipped with an argon/krypton laser with x63 oil objective and 1.4 aperture (Leica Microsystems). Green fluorescence was detected at $\lambda > 515$ nm after excitation at 488 nm. For dual analyses, green fluorescence was detected at 520–560 nm. Red fluorochromes were excited at 568 nm and detected at $\lambda >$ 585 nm. All experiments presented were repeated several times on different days and results were consistent and reproducible. Further image processing was performed using the ImageJ software package.

For EM, cells were allowed to take up beads or bacteria for 40 min or 4 h (to study MIIC-SCV fusion) and fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%). After embedding in a mixture of methyl cellulose and uranyl acetate, ultrathin sections were stained and analyzed with a Philips CM10 electron microscope.

Plating Assay

For enumeration of intracellular surviving bacteria, freshly isolated primary B cells were incubated with anti-IgM coated *Salmonellae* and Ramos cells with uncoated *Salmonellae* as a control, washed, and cultured in medium with 10 µg/ml gentamycin as described above. At the indicated time points, cells were washed with PBS and lysed in 0.1% Triton X-100 (Merck) for 10 min on ice, washed with PBS, and a dilution series was plated onto LB agar plates. Plates were incubated overnight at 37°C and colonies were counted.

ELISA assays

To determine IgM levels in culture supernatants, flat-bottom Maxisorb plates (Nunc) were coated with polyclonal anti-IgM (SH15; Sanquin) in 100 µl of PBS (pH 7.4; NPBI International) overnight at room temperature. Plates were washed with PBS/0.02%Tween 20 (Mallinckrodt Baker) and samples were incubated for 2 h in

high-performance ELISA buffer (Sanquin). As a standard, pooled human serum was used. Plates were washed and incubated for 1 h with 1 µg/ml mAb anti-human IgM-HRP (MH15-HRP; Sanquin).

Whole-cell *Salmonella* ELISA was performed by coating overnight at 37°C of *Salmonella* to Maxisorb plates in 100 µl 0.1 M sodium bicarbonate at pH 9.6 supplemented with 10 µg/ml gentamycin. Plates were washed extensively with PBS/0.02%Tween-20 and supernatants were incubated in high-performance ELISA buffer. Plates were washed and incubated for 1 h with 1 µg/ml mAb anti-human IgM-HRP (MH15-HRP, Sanquin).

After washing, peroxidase activity was visualized by incubation with 100 µl 3,5,3',5'-tetramethylbenzidine (Merck), 100 µg/ml in 0.11 M sodium acetate (pH 5.5), containing 0.003% H_2O_2 (Merck). The reaction was stopped by addition of an equal volume of 2 M H_2SO_4 (Merck) and the absorbance at 450 and 540 nm was measured immediately in a Titertek plate reader. Results were calculated with LOGIT software (http://www.xs4all.nl/~ednieuw/Logit/logit.htm).

Statistical analysis

Statistical significance was determined using the Mann-Whitney *U* test.

Results

Efficient BCR-mediated phagocytosis of large particulate Ags by B cells

Unlike other professional Ag presenting cells, primary B cells show very limited phagocytic capacity. Ag uptake by B cells is critically dependent on the selectivity of the B cell receptor (BCR) (10, 28). The current view on BCR-mediated Ag uptake by B cells mainly centers on soluble Ags like small foreign proteins or shedded bacterial coat products (29). Accordingly, most B cell activation studies involve the global triggering of BCR using soluble cross-linking Ags rather than pathogen-associated Ags. We opted to study BCR-mediated recognition of particulate Ags in B cells by inducing localized clustering of the BCR using beads decorated with mAb against the BCR. When anti-IgM coated beads contacted a Ramos B cell stably expressing MHC class II HLA-DR1 tagged with GFP (DR-GFP, which localizes to the plasma membrane and lysosomal MIIC vesicles), rapid and efficient internalization of the bead ensued (Fig. 1*A* and supplemental movie 1). Ramos cells polarized themselves toward the contact site during uptake of the beads such that the nucleus moved to the side opposite of the bead, analogous to the situation

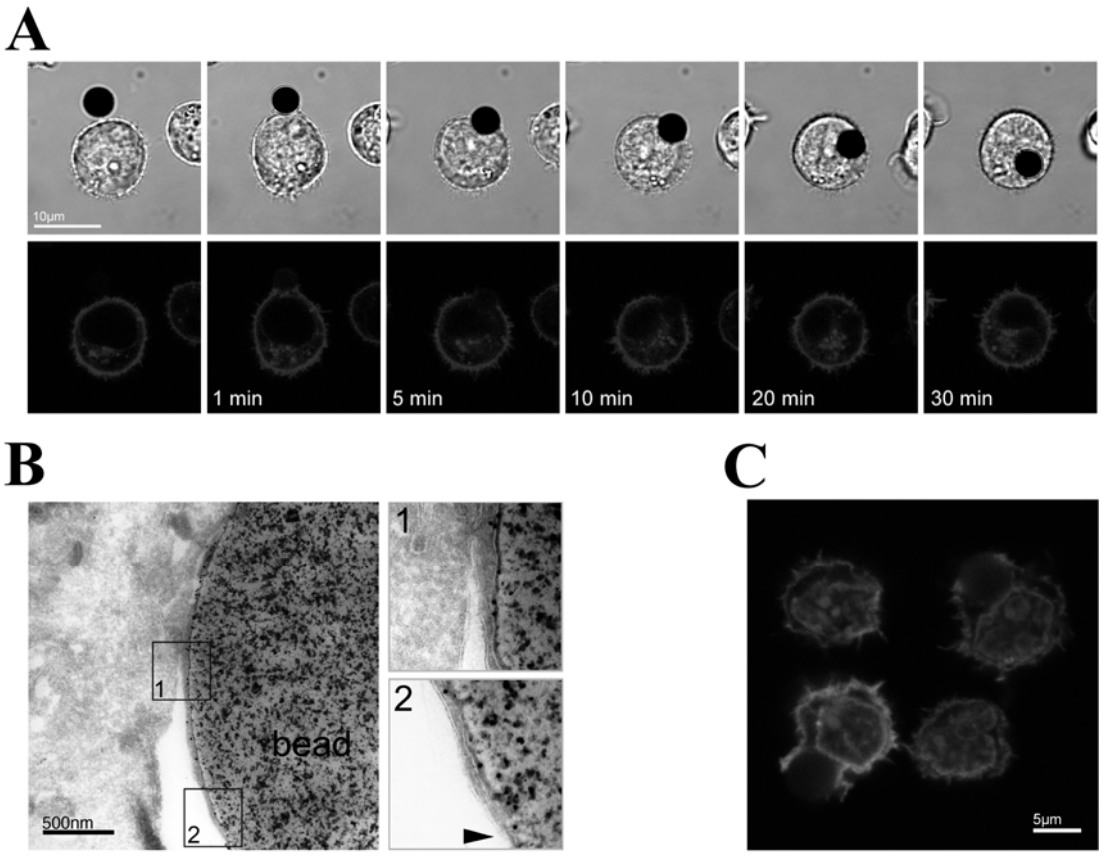


Figure 1. Efficient BCR-mediated phagocytosis of large particulate Ags.
(*A*) Living Ramos B cells expressing DR-GFP and anti-IgM-coated beads were imaged every 30 s. Depicted are time points after initial contact, top panel: transmission image, bottom panel: GFP signal. Scale bar = 10µm. Images are frames from supplemental movie 1. (*B*) Ramos cells were fixed 10 min after addition of anti-IgM coated beads and analyzed by cryoelectron microscopy. Scalebar = 500nm. Zoom-ins of the thin membrane extrusions surrounding the bead are shown for indicated regions. The tip of the protrusion is indicated with an arrowhead in inset 2. (*C*) Cells were fixed 10 min after addition of anti-IgM coated beads and processed for immunofluorescence confocal microscopy. Depicted is the overlay of the signals from DAPI nuclear staining (blue), phalloidin-stained actin-cytoskeleton (red), and DR-GFP (lower left cell only). Scalebar = 10µm. Figure represents one section from a Z-stack. A three-dimensional reconstruction is provided as supplemental movie 4.

following Th cell contact (30) or following cytotoxic CTL-target cell interactions (31). Internalization reached completion within 10-20 min and required an intact cytoskeleton (as the microtubule-disruptive agent nocodazole prevented phagocytosis, supplemental movie 2). In addition, uptake was BCR dependent because beads coated with an irrelevant Ab were not internalized (supplemental movie 3). Ramos cells do not express FcγRs, which excludes their involvement in bead uptake. A detailed analysis by cryoelectron microscopy revealed some of the

impressive cellular events underlying uptake of large particulate Ags. During the initial phase of contact, Ramos cells surrounded the bead with a surprisingly thin double membrane originating from the cell surface (Fig. 1*B*). Staining with phalloidin of Ramos cells in the process of bead phagocytosis revealed extensive actin fibers in the membrane protrusions surrounding the bead (Fig. 1*C* and supplemental movie 4). Together, these data show that B cell are able to internalize inert particles in a process that fulfills the criteria of phagocytosis. Thus, different from the general concept that primary B cells are essentially nonphagocytic cells, B cells are very efficient phagocytes when particle recognition is facilitated by the BCR.

B cell lines can internalize* Salmonella *via their BCR

We generated a stable transfectant of the Ramos B cell line with the MHC class II Ag presentation chaperone HLA-DO tagged with GFP (DO-GFP). DO-GFP localizes to the MIICs in living cells. As the Ag specificity of the BCR of Ramos cells is unknown, we coated the bacteria with anti-IgM-anti-LPS tetrameric Ab complexes. Within 1 mi after first contact, Ramos cells efficiently internalized GFP-*Salmonella* coated with tetrameric Ab complexes (Fig. 2*A* and supplemental movie 5). Uncoated *Salmonella* were ignored by Ramos B cells (supplemental movie 6), showing that *Salmonella* by itself is not able to invade the Ramos B cell line. To investigate the internalization process in more detail, we used cryoelectron microscopy on Ramos cells shortly after encounter of the coated bacteria. This showed the formation of a phagocytic cup and the extension of pseudopodia around the bacteria, demonstrating that B cells actually seem to phagocytose the *Salmonella* bacteria (Fig. 2*B*). To study the interaction between the green MIICs in Ramos DO-GFP with the SCVs, we used a red mRFP-*Salmonella*. Ramos DO-GFP cells incubated with coated mRFP-*Salmonella* showed bacterial uptake and rapid translocation of the MIICs to the SCVs (Fig. 2*C* and supplemental movie 7). Multiple intimate contact events were observed between the membrane of the SCVs and the DO-GFP positive MIICs, suggesting fusion events of the MIICs with the SCV immediately after bacterial uptake.

To study the acquisition of MHC class II molecules on the SCV membrane, we used Ramos cells expressing DR-GFP. MHC class II molecules already localized to the membrane of the SCV during the actual process of BCR-mediated uptake of coated mRFP-*Salmonella* (Fig. 2*D* and supplemental movie 8). Similar to the DO^+ vesicles,

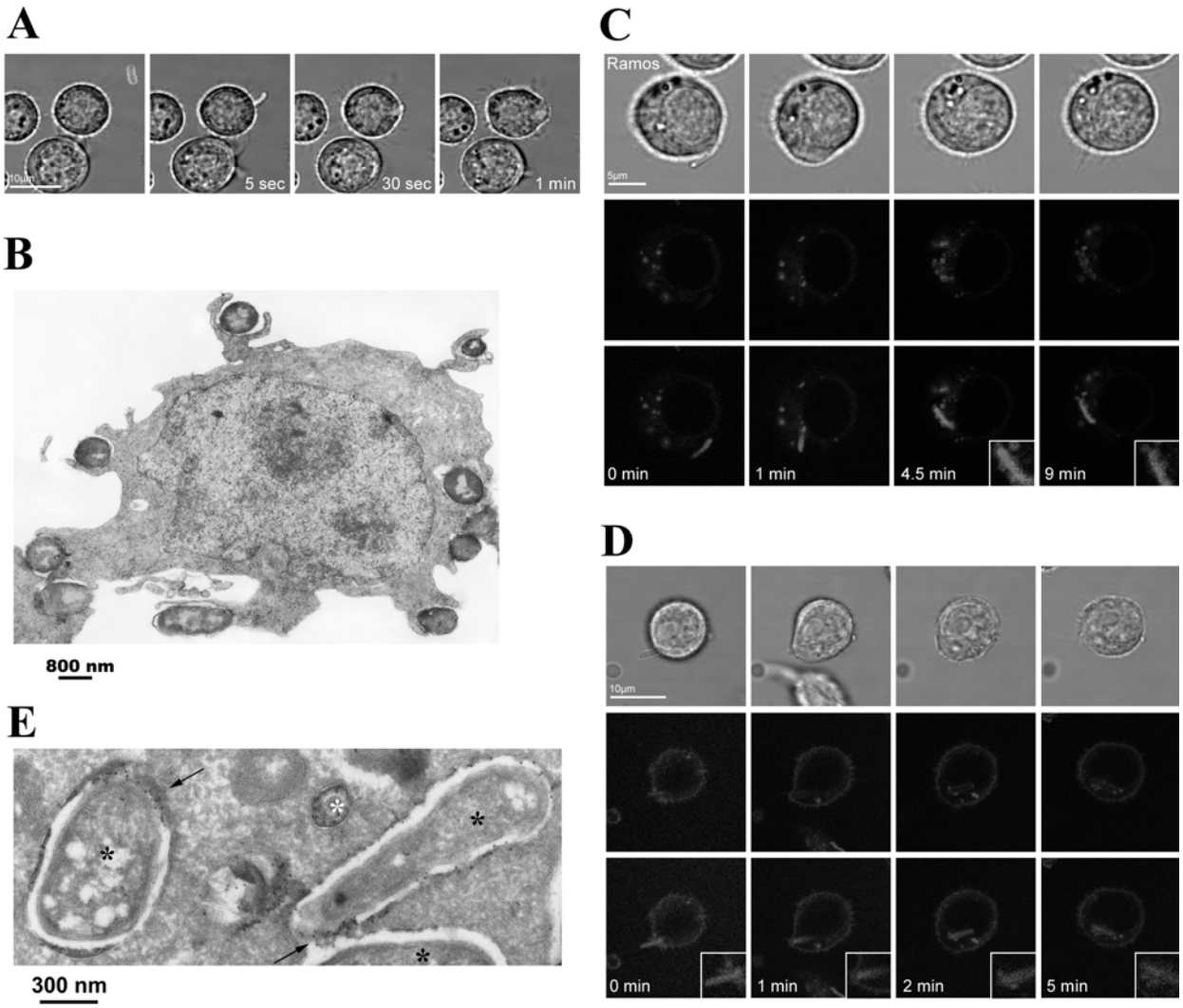


Figure 2. Efficient BCR-mediated internalization of *Salmonella*.

(*A*) Living Ramos cells, expressing DO-GFP, and GFP-expressing *Salmonella* were imaged every 3 s. Depicted are time points after initial contact. GFP signal is projected on top of the transmission image. Scale bar = 10µm. Images are frames from supplemental movie 5. (*B*) Electron microscopic analysis of Ramos cells in the process of phagocytosing anti-BCR-coated *Salmonella*. Scale bar = 800nm. Note the cup-shaped pseudopodia of the B cell at contact places with the bacteria. (*C*) Living Ramos cells expressing DO-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are time points after initial contact, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scale bar = 5µm. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 7. (*D*) Living Ramos cells expressing DR-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are indicated time points after initial contact, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scale bar = 5µm. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 8. (*E*) Immunoelectron microscopic analysis of primary B cells with internalized anti-BCR coated *Salmonella* and CD63 (10-nm gold particles). Black asterisks mark bacteria, the white asterisk marks an MIIC; and arrows indicate fusion events with MIICs.

we observed extensive docking of DR-GFP$^+$ vesicles with the SCV membrane within minutes after entry. Because molecular exchange between the MIIC vesicles and the SCVs is critical for generation of MHC class II molecules complexed with *Salmonella* Ags, we visualized MIIC-SCV fusion by EM. Indeed, fusion between the characteristic multivesicular MIICs and the SCVs was frequently observed. Immunostaining showed that, next to the MIICs, the SCV membrane stained positive for CD63 (Fig. 2*E*). Quantification of the fusion events between MIICs and 100 SCVs showed that in 10% of the SCVs the actual process of MIIC-SCV fusion was captured in the time frame of cell fixation. Thus, the combined acquisition of MHC class II on the SCVs and the frequent fusion events between SCVs and MIICs generates all conditions required for Ag presentation of *Salmonella* Ags.

BCR-mediated internalization of* Salmonella *by primary human B cells

Since Ramos cells are at least 1.5 times larger than primary B cells, we tested whether primary B cells could internalize *Salmonella* as well. To distinguish between binding of bacteria to the BCR and actual uptake, we used a mAb against *Salmonella*-LPS. Completely internalized GFP-positive bacteria will not be stained, while extracellular and partially engulfed bacteria will be accessible to the anti-LPS antibody. Incubation of primary human B cells with uncoated GFP-*Salmonella* consistently revealed a small but significant population of B cells (4.3%, SD = 1.1, n = 6) that recognized and internalized the native bacterium via direct recognition of *Salmonella* Ags by the B cell's BCR (Fig. 3*A*). A similar proportion of primary B cells recognized and captured dead uncoated GFP-*Salmonella* via their BCR (4.1%, SD = 1.5, n = 4), but failed to internalize dead *Salmonellae* since these B cells stained all positive for LPS (Fig. 3*A*). Analysis by confocal microscopy confirmed that internalized viable *Salmonellae* are completely taken up by primary B cells, resulting in one to three intracellular bacteria per B cell (Fig. 3*B*, left *panel*). Incubation with fixed bacteria only showed binding but no uptake of *Salmonella* (Fig. 3*B*, right *panel*). To address the possible involvement of FcγRs on primary B cells, we preincubated primary B cells with F(ab)$_2$ fragments of the anti-IgM antibody MH15 to block the internalization of anti-IgM coated *Salmonella* by primary B cells. This resulted in inhibition of 80%, illustrating that internalization is indeed BCR-mediated (supplemental Fig. 1). Ideally, we would also like to block BCR-mediated internalization of *Salmonella* via direct recognition of *Salmonella* Ags by the BCR. However, since blocking of the Ag binding site of the BCR is impossible

due to lack of anti-Id Abs, we studied the effect of BCR internalization before addition of the bacteria on the efficiency of bacterial uptake. We combined Abs against the H chain of IgM with cross-linking goat-anti-mouse antibodies. This resulted in a partial internalization of the BCR (mean fluorescence intensity for membrane-bound IgM dropped from 3767 to 2275) and a concomitant reduction in GFP^+/LPS^- B cells that had completely internalized *Salmonella* (supplemental Fig. 2). In addition, Ramos cells efficiently internalized GFP-*Salmonella* in a BCR dependent manner only when *Salmonella* was coated with anti-IgM (Fig. 3*C*). No GFP-positive Ramos cells were found without the anti-IgM coat, showing that *Salmonella* is unable to infect Ramos cells autonomously. As a control for IgM-type BCR-mediated uptake of Ramos, *Salmonella* coated with anti-IgG were incubated with Ramos cells, and (similar to uncoated bacteria) these were not internalized. Incubation of the IgG-type BCR-expressing B cell line Cess with anti-IgG-coated bacteria showed that the anti-IgG coated-bacteria were efficiently taken up by Cess (data not shown). Up to 90% of the Ramos cells acquired one or more anti-IgM coated bacteria. However, ~ 25% of Ramos cells contained internalized GFP-*Salmonella* only, while >60% of Ramos cells were also positive for LPS staining. Confocal microscopy of cells labeled by anti-LPS antibodies showed that these represented Ramos cells that had internalized some but not all bound bacteria (data not shown).

Since dead bacteria were not internalized, *Salmonella* may be requiring both recognition by BCR and bacterial-mediated processes to enter primary human B cells. *Salmonella* can invade host cells by expressing type III secretion systems encoded either by *Salmonella* pathogenicity island (SPI)-1 to translocate effector proteins into host cell cytoplasm that trigger internalization of the bacteria or by SPI-2 to modulate intracellular trafficking and replication of *Salmonella* within a modified vacuolar compartment. Recent studies however have modified this concept to some extent as they show a partial overlap in SPI-1 and SPI-2 functions (32). To address involvement of these type III secretion systems, we used *Salmonella* defective in SPI-1 (invA mutant) or SPI-2 (ssrA mutant). Incubation of primary B cells with SPI-1 defective *Salmonella* showed a reduction in internalized bacteria and no bacteria adhering to primary B cells. Incubation with SPI-2-defective *Salmonella* showed a milder reduction in internalized bacteria and a minimal reduction in adhering bacteria (Fig. 3*D*). This indicates that SPI-1 is involved in attachment to primary B cells in conjunction with the BCR. Components

of SPI-1, and to a lesser extent SPI-2, are involved in BCR-mediated uptake of *Salmonella* by primary B cells.

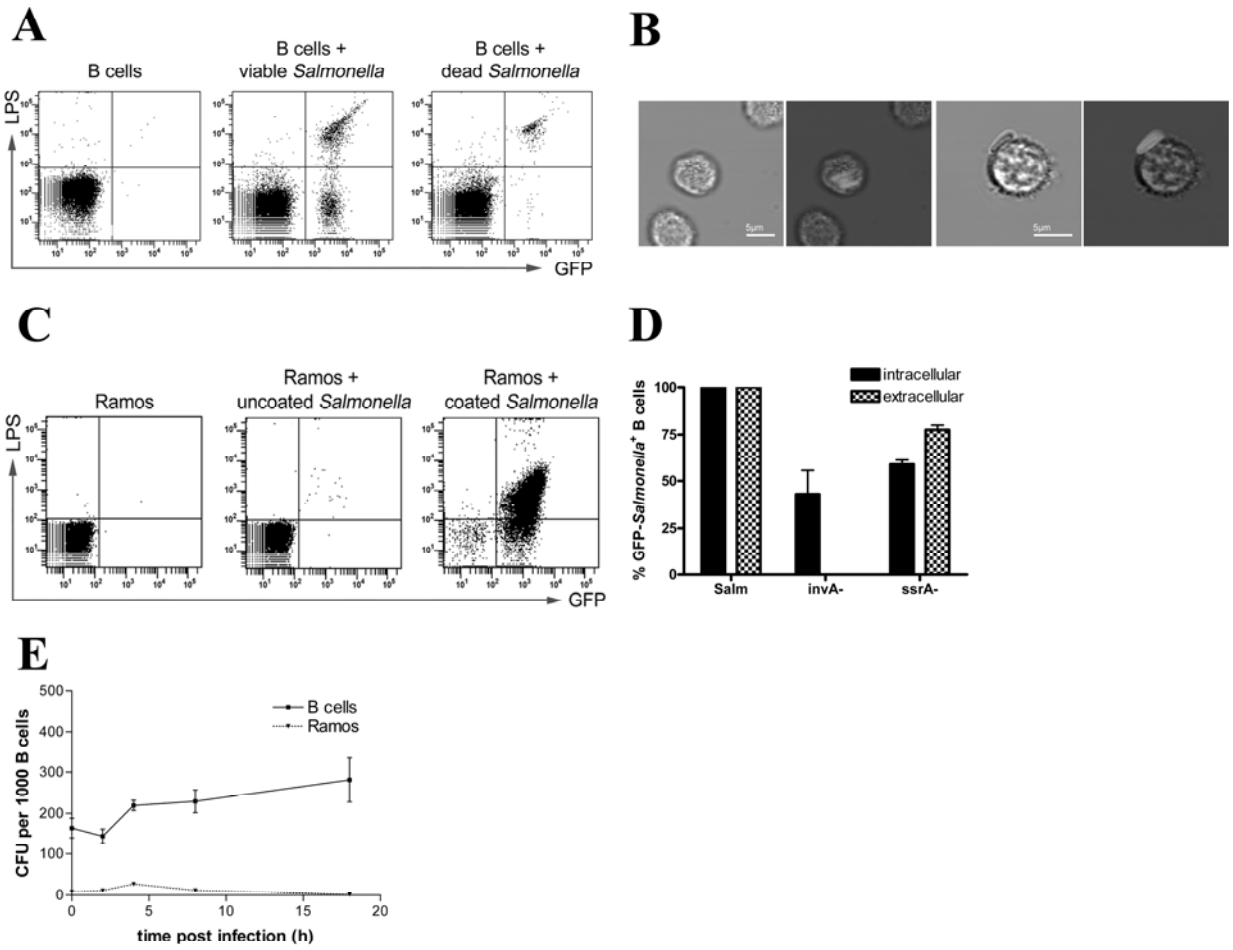


Figure 3. BCR-mediated internalization and survival of *Salmonella* in primary B cells.
(*A*) CD19$^+$ B cells were incubated with viable GFP-*Salmonella* for 40 minutes and FACS analyzed. Anti-LPS-APC versus GFP scatter plots are depicted. (*B*) Living primary human B cells were incubated with viable (*left*) or dead (*right*) GFP-*Salmonella* for 40 min and imaged using confocal microscopy. Left panel: transmission image, right panel: GFP signal projected on transmission image. Scalebar = 5µm. (*C*) Ramos B cells were incubated with viable GFP-*Salmonella* for 40 min, stained with an antibody against *Salmonella* LPS and analyzed by FACS. Depicted are anti-LPS-APC vs. GFP scatter plots of 50.000 events. (*D*) CD19$^+$ B cells were incubated with viable wild-type GFP-*Salmonella* or mutant for SPI-1 (invA$^-$) or SPI-2 (ssrA$^-$) for 40 minutes and analyzed by FACS. Intracellular indicates B cells that are GFP$^+$LPS$^-$ and extracellular indicates GFP$^+$LPS$^+$ B cells. Data are from two independent experiments with cells from four different donors, error bars indicate SEM. (*E*) B cells were incubated with anti-IgM-coated *Salmonella* and Ramos cells with uncoated *Salmonellae*, lysed and plated onto LB-agar plates. Data are from duplicates of experiments performed with B cells from two individual donors, error bars indicate SEM.

Phagocytosed *Salmonella* grow in many cell types but can efficiently be destroyed in specialized cells like macrophages and neutrophils (33). To examine survival of internalized *Salmonella* in primary B cells, we performed plating assays. B cells were incubated with anti-IgM coated *Salmonella* and washed and cells with internalized bacteria were followed in time. At different time points, cells were lysed and intracellular bacteria were plated onto agar. Internalized *Salmonella* remained vital for the 18 h that we tested (Fig. 3*E*). Ramos cells incubated with uncoated *Salmonella* were used as a control, since uncoated *Salmonella* are not taken up by Ramos cells (supplemental movie 6). Indeed, hardly any *Salmonellae* were recovered after incubation with Ramos (Fig. 3*E*). Thus, *Salmonella* survives intracellularly after BCR-mediated internalization by primary B cells.

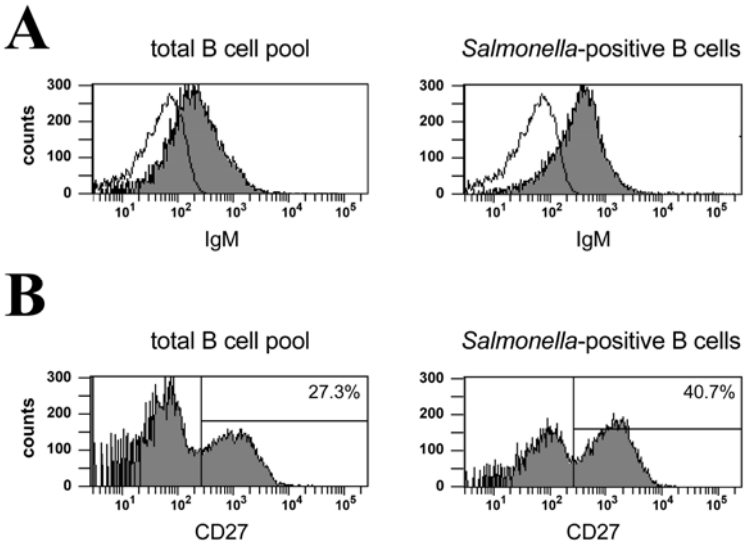


Figure 4. Both naive and memory B cells internalize *Salmonella*.
(*A*) Primary B cells were incubated with GFP-*Salmonella* and stained for IgM before and after FACS sorting. The open histogram represents the IgG1 isotype control. (*B*) The cells from 3*A* were analyzed for expression of CD27. Percentages of $CD27^+$ B cells are given. For IgG1 isotype control, see Fig. 3*A*.

Which peripheral B cell type is able to internalize *Salmonella*? Most peripheral B cells express the IgM surface Ig receptor (34). To confirm that internalization of *Salmonella* occurs via the BCR, we analyzed IgM expression on *Salmonella*-containing B cells. This showed that B cells that have internalized *Salmonella* expressed surface IgM (Fig. 4*A*). The mean fluorescence intensity of IgM for the total B cell pool is 478 and for the *Salmonella*-containing B cells 825. Two major subsets of B cells can be identified in adult peripheral blood according to the expression of CD27. CD27 expressing B cells comprise memory B cells while CD27-

negative B cells represent naive and transitional B cells (35). FACS analysis showed that IgM memory B cells ($CD27^+$) internalized *Salmonella* more efficiently than IgM^+CD27^- naïve B cells (Fig. 4*B*). Although a proportion of the naive IgM^+ B cells are able to take up *Salmonella*, *Salmonella* is preferentially internalized by the circulating IgM^+ memory B cells.

Presentation of BCR-internalized Ags by B cells to T cells

When *Salmonella* survives within the phagosome (see Fig. 3*E*) following BCR-mediated internalization, does this result in MHC class II mediated presentation of *Salmonella* Ags? To test this, primary human B cells were incubated with anti-IgM-coated *Salmonellae* to achieve BCR-mediated uptake by all IgM^+ B cells and maximize the number of Ag-presenting B cells. These cells were subsequently cultured in the presence or absence of autologous primary T cells. After 5 days, [^{3}H]thymidine was added and cells were harvested after 18 h. Incubation of B cells with anti-BCR-coated *Salmonella* induces proliferation of the B cells (green line, Fig. 5*A*), demonstrating that BCR ligation and BCR-mediated internalization of *Salmonella* effectively activated B cells. B cells incubated with coated *Salmonella* and cultured with autologous T cells results in an Ag-specific proliferation of T cells (red line) (Fig. 5*A*).

To study whether primary B cells with a BCR directed against *Salmonella* also induce T cell proliferation, we incubated uncoated *Salmonella* with primary B cells (Fig. 5*B*, *left panel*). Addition of autologous T cells yielded a *Salmonella*-specific T cell proliferation response (blue vs pink line). B cells incubated with uncoated dead *Salmonella* that could not be internalized were able to induce T cell proliferation (Fig. 5*B*, *right panel*). However, T cell proliferation is optimal when viable *Salmonellae* have been internalized by B cells (Fig. 5*B*: *left* vs *right panel*). Incubation of only T cells with uncoated or coated bacteria did not result in B cell-independent proliferation of the T cells (data not shown).

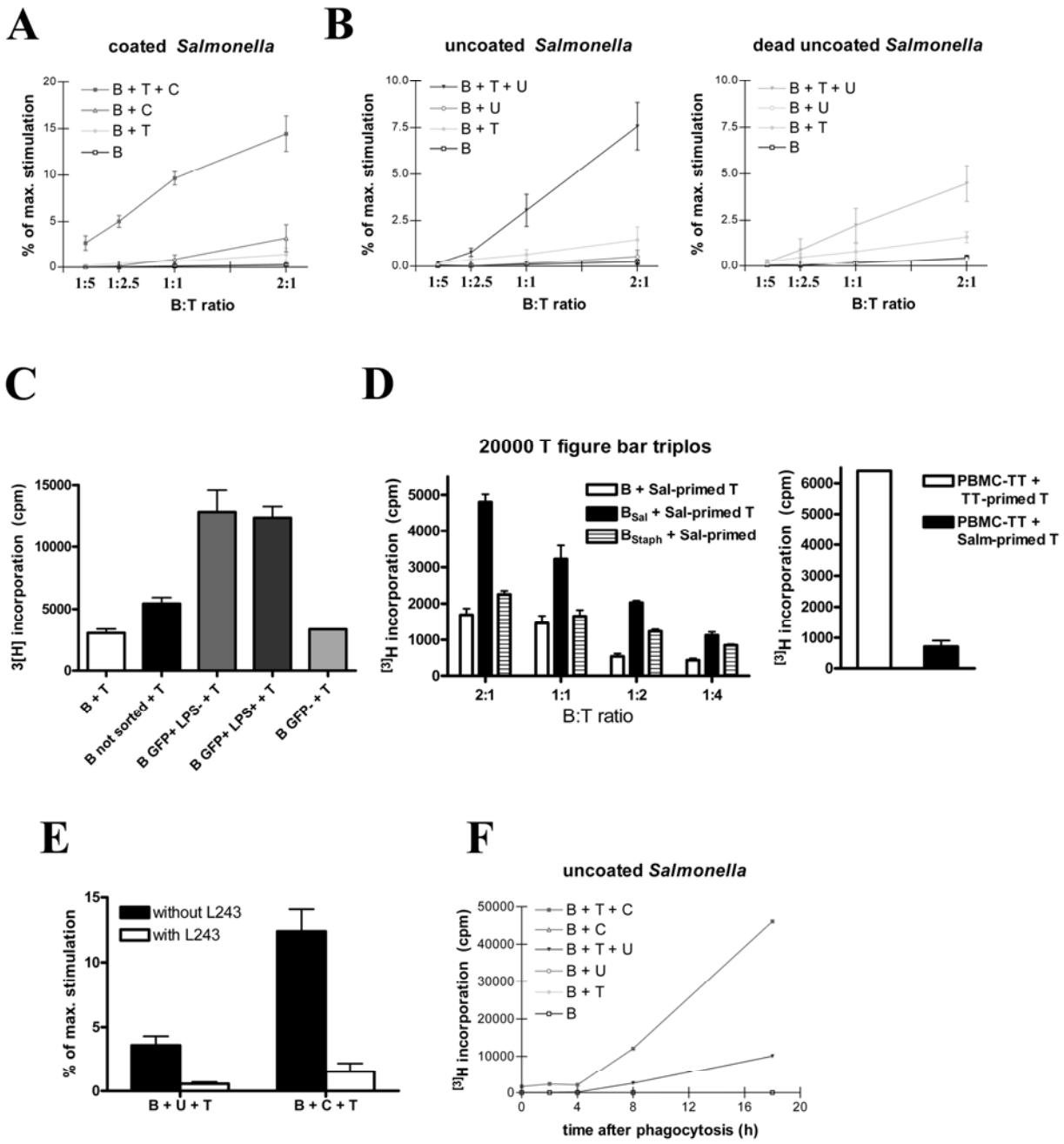
To demonstrate that the T cell proliferation in Fig. 5*B* was indeed induced by the fraction of B cells that had captured *Salmonella*, we FACS sorted the GFP-*Salmonella*-positive B cells in fractions positive and negative for anti-LPS staining and cultured these with autologous T cells. T cells only proliferated when cultured with B cells that had captured GFP-*Salmonella* (Fig. 5*C*). It may be that complete internalization is not required for Ag presentation but that only capturing of the bacteria by the BCR suffices.

We can however not exclude that GFP-*Salmonella*$^{+}$LPS^{+} B cells also contain completely internalized bacteria as we have shown that B cells are able to take up more than one *Salmonella*. Moreover, even though *Salmonella* survives in the vacuole and suppresses MHC class II Ag presentation in phagosomes (27), *Salmonella* Ags are still efficiently presented by B cells. This probably reflects Ag degradation and loading on MHC class II molecules in normal MIICs after content exchange between phagosome and MIICs due to the observed intimate contact and extensive fusion events.

To demonstrate the Ag-specificity of the proliferating T cells, we performed restimulation assays in which we sorted the T cells that proliferated in response to B cells that had taken up *Salmonella*, and restimulated these *Salmonella*-primed T cells with autologous B cells that had taken up *Salmonella* or *Staphylococcus*. This showed that the *Salmonella*-primed T cells are indeed for the large part Ag specific, as they proliferate better in response to B cells that had internalized *Salmonella* than B cells that had internalized *Staphylococcus* or control B cells without bacteria. As a control, T cells primed against *Staphylococcus* did proliferate in response to B cells that had internalized *Staphylococcus*, demonstrating that these B cells did present *Staphylococcus* Ags to CD4+ T cells (Fig. 5*D*, *left panel*). In addition, we restimulated *Salmonella*-primed T cells with PBMCs presenting tetanus toxoid Ags. This showed no response of the *Salmonella*-primed T cells, while tetanus toxoid-primed T cells from the same donor proliferated after restimulation (Fig. 5D, *right panel*).

Furthermore, we performed blocking assays to show that the T cell response depends on the MHC class II Ag presentation pathway with L243, an Ab that blocks the MHC class II-TCR interaction (Fig. 5*E*). The induction of T cell activation depended on presentation of *Salmonella* Ags via MHC class II (HLA-DR), as the T cells failed to respond after blocking of MHC class II with L243.

The observation that fusion of MIICs with the phagosome occurs swiftly prompted us to examine whether this had consequences for Ag presentation. When we irradiated B cells immediately after incubation with anti-IgM coated *Salmonella*, no proliferation of B or T cells was observed after 6 days. B cells apparently need to be viable to process and present *Salmonella* Ags to T cells. To study the kinetics of Ag presentation, B cells were incubated with native or anti-IgM coated *Salmonella* and B cells were irradiated at several time points before incubation with T cells. After 5 days, [^{3}H] thymidine was added and cells were harvested after 18 h. Noninfected B



cells did not induce T cell proliferation after irradiation. Anti-BCR coated *Salmonella* internalized by IgM^+ B cells start to induce proliferation of T cells immediately (red line) and uncoated *Salmonella* internalized by BCR-reactive B cells 4 h after uptake of the *Salmonella* (blue line) (Fig. 5*F*). Ag presentation thus starts at times corresponding to the earliest phases of BCR-induced internalization and rapid fusion with the MIICs. Primary B cells rapidly present Ags of internalized *Salmonella*, even if the bacterium survives inside a B cell.

Figure 5. BCR-mediated uptake of *Salmonella* induces Ag presentation by B cells.
(*A*) BCR-induced internalization results in proliferation of B cells and Ag-specific T cells. B cells (B) were either or not incubated with viable anti-BCR-coated (C) *Salmonella* in the presence or absence of autologous T cells (T), as indicated. Results are shown as percentage of maximal stimulation of T cells with anti-CD3 and -CD28 Abs. (*B*) The same experimental setup as in Fig. 4*A* was performed with uncoated (U) viable and dead *Salmonellae*. Data are from four independent experiments of different donors, error bars indicate SEM. B:T represents the ratio of different amounts of B cells added to a fixed amount of T cells. Experiments with uncoated and coated *Salmonella* were performed in parallel using the same donor. (*C*) B cells incubated with viable uncoated GFP-*Salmonella* were FACS-sorted as indicated and incubated for 6 days with T cells. White bar: B cells without bacteria, black bar: B cells before sorting, green bar: GFP-*Salmonella*$^{+}$LPS^{-}-sorted B cells, blue bar: GFP-*Salmonella*$^{+}$LPS^{+}-sorted B cells and grey bar: GFP-*Salmonella*$^{-}$-sorted B cells. Data are from two independent experiments with cells from different donors, error bars indicate SEM. (*D*) *Salmonella*-primed or *Staphylococcus*-primed T cells were restimulated for 2 days with autologous B cells that were incubated with viable *Salmonella* or *Staphylococcus* or restimulated with PBMCs incubated with tetanus toxoid. Data are representative for three independent experiments with different donors, error bars indicate SEM. (*E*) T cells (T) were cultured with B cells (B) that had taken up uncoated (U) or anti-BCR-coated (C) *Salmonella* either or not in the presence of the MHC class II Ag presentation blocking Ab L243. (*F*) Ag presentation by B cells starts immediately after internalization of the *Salmonella*. B cells (B) were either or not incubated with uncoated (U) or anti-BCR-coated (C) *Salmonella* and irradiated with 60 Gy at different time points before T cells (T) were added. Data are representative for four independent experiments of different donors.

BCR-mediated internalization induces IgM secretion

To test if BCR-mediated internalization of *Salmonella* leads to differentiation of B cells into Ab-secreting cells, supernatants of B cells that internalized bacteria were tested for the presence of human IgM after culture. After 5 days incubation with viable uncoated bacteria, no strong induction of IgM secretion following BCR-mediated internalization was detectable (Fig. 6*A*, *left panel*). When the *Salmonella* were coated with anti-IgM Abs, B cells produced four times more IgM than uncoated bacteria. Addition of T cells did not increase IgM production in the first 5 days, indicating that IgM production resulted from a T cell-independent activation of B cells (Fig. 6*A*, *left panel*). T cell help did occur within 12 days, leading to a strong increase in IgM production (Fig. 6*A*, *right panel*). Thus, BCR-mediated internalization of *Salmonella* induces autonomous IgM secretion by B cells, whereas T cell help is required during the late stage of Ig secretion by B cells. IgG production of B cells incubated with *Salmonella* did not significantly surpass IgG production levels from B and T cells that were not incubated with *Salmonella* (data

not shown). This is in line with the observation that the B cells that take up *Salmonella* are IgM^+ memory B cells. It also indicates that BCR-mediated internalization of *Salmonella* by the naive IgM^+ B cell pool does not induce Ig class switching under our culture conditions.

When B cells that internalize *Salmonella* through the BCR are activated, they might produce *Salmonella*-specific Abs. We incubated uncoated, viable GFP-*Salmonella* with primary B cells and FACS-sorted the GFP^+ B cells. We cultured the sorted B cells on a monolayer of fibroblasts expressing human CD40L to provide costimulation. After 12 days, total human IgM as well as *Salmonella*-reactive Abs were quantified. B cells that internalized *Salmonella* produced more total IgM than B cells that did not take up *Salmonella* (Fig. 6*B*, *upper left panel*). The production of *Salmonella*-reactive Abs was measured using a whole-cell *Salmonella* ELISA.

Strikingly, the sorted *Salmonella*-containing B cells produced significant amounts of anti-*Salmonella* IgM (Fig. 6*B*, *lower left panel*), unlike control B cells from the same donor. Correction of the anti-*Salmonella* reactive IgM for total IgM production by the B cells revealed that the sorted B cells produced significantly higher levels of anti-*Salmonella* IgM compared to control B cells (Fig. 6*B*, *right panel*). The production of significantly higher levels of anti-*Salmonella* IgM clearly shows the involvement of the BCR in internalization of *Salmonellae*. If the BCR would not be involved, but bacterial internalization and subsequent B cell activation would solely depend on TLR stimulation and/or co-stimulation, the B cells that had taken up *Salmonella* would not be able to produce *Salmonella*-specific IgM. Thus, BCR-mediated internalization of *Salmonella* forms an efficient pathway to induce differentiation of *Salmonella*-specific B cells and production of *Salmonella*-reactive IgM Abs.

Discussion

B cells may encounter Ags as free Ag or delivered by Dendritic cells (DCs) (36). DCs are equipped with both nondegradative and degradative Ag uptake pathways to facilitate Ag presentation to both B and T cells. Blood DCs can capture and transport particulate Ags such as invading bacteria to the spleen, where they promote differentiation of marginal zone B cells into IgM secreting plasma cells (37). We here show a pathway independent of DCs and macrophages. In contrast to the dogma that mammalian B cells lack the ability to ingest pathogens and are only involved in the adaptive phase of the immune response (38) or that entry of

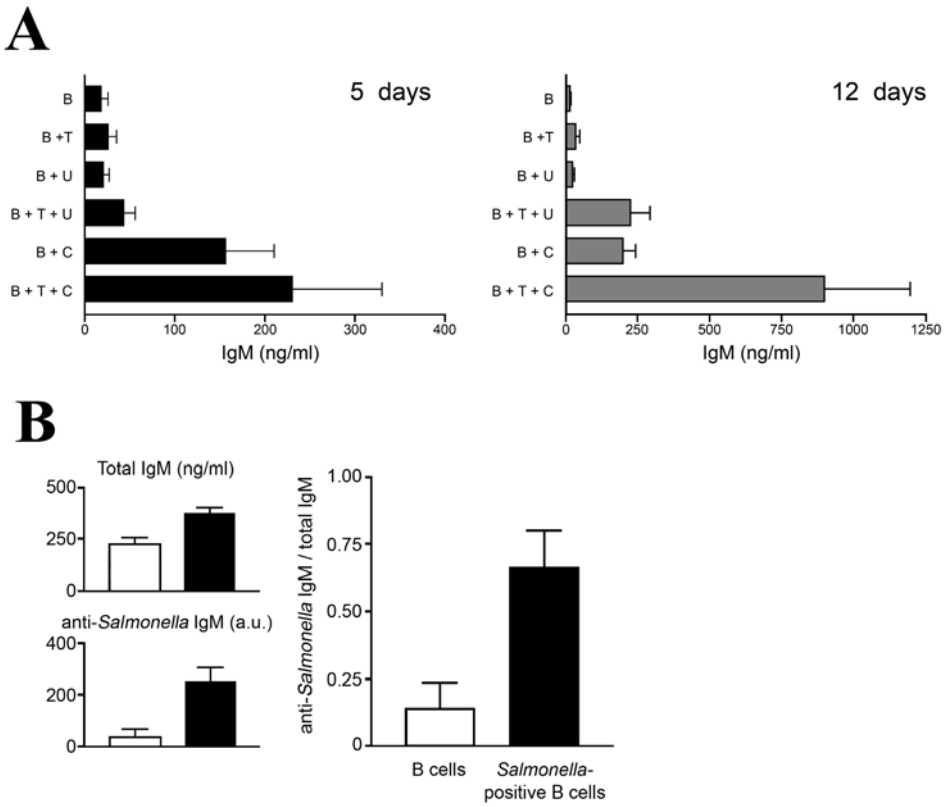


Figure 6. BCR-mediated internalization induces IgM production and B cells with a BCR reactive for *Salmonella* Ags produce anti-*Salmonella* IgM.

(*A*) B cells (B) were either or not incubated with uncoated (U) or anti-BCR coated (C) *Salmonella* in the presence or absence of autologous T cells (T). After 5 (black bars, *left panel*) and 12 days (grey bars, *right panel*) total secreted human IgM was determined. (*B*) B cells either or not incubated with viable uncoated GFP-*Salmonella* were FACS sorted and cultured on a monolayer of irradiated, CD40L-expressing fibroblasts for 12 days and supernatant was analyzed for total IgM production and *Salmonella*-reactive IgM production. White bars represent B cells and black bars represent *Salmonella*-positive B cells. Anti-*Salmonella* IgM was divided on the total amount of IgM measured in the supernatants (*right panel*, $P = 0.006$). Data are representative for three independent experiments of different donors.

Salmonella in B cells is a random process (39), primary B cells can internalize *Salmonella* via their specific BCR. So far, the general concept for Ag presentation of bacterial peptides by B cells was that B cells extract proteins from the surface of DCs or bacteria or bind shedded bacterial proteins (15). Indeed, this may occur for dead or lysed bacteria killed by Abs and complement or after antibiotic treatment. Our observation that recognition via the BCR of dead bacteria without internalization induces Ag presentation to T cells is in line with this concept. However, internalization of viable bacteria leads to superior $CD4^+$ T cell activation and instantaneous generation of anti-*Salmonella* Abs by autonomous activation of the *Salmonella*-reactive B cells.

It has been proposed that Abs made by IgM memory B cells are the first-line defense mechanism against all infections and that Abs produced by IgM memory B cells are the only B cell defense against T-independent Ags (40). IgM^+ memory B cells in peripheral blood represent circulating splenic marginal zone B cells in charge of T-independent responses (41). Since marginal zone B cells express a BCR of polyreactive nature (42), this could explain the relatively high numbers of $CD27^+$ B cells that take up *Salmonella*. As for IgM^+ memory B cells, a subset of mature naive B cells in peripheral blood are polyreactive (43). Combined, the primary B cells that we found to internalize *Salmonella* seem to represent naive and IgM^+ memory B cells with a polyreactive BCR.

How do these findings relate in the involvement of B cells in *Salmonella* infection? Studies in B cell-deficient mice show that B cells are necessary for efficient protection against both primary and secondary infection with *Salmonella* (44). Passive transfer of *Salmonella*-immune serum could not restore resistance of mice to *Salmonella* (45), demonstrating that high-affinity Ab production alone is not the only function of B cells in salmonellosis. Moreover, at the early stage of primary infection class-switched high-affinity Abs against *Salmonella* are not yet available and cannot explain the importance of B cells at this stage. Therefore polyreactive, IgM^+ memory B cells may well be involved in protection against primary infection via BCR-mediated internalization of *Salmonella* and rapid generation of protecting *Salmonella*-reactive IgM Abs. B cell deficient *Igh-6*$^{-/-}$ mice have impaired Th1 T-cell responses from the early stage of *Salmonella* infection, showing that B cells play an essential role in the initiation of T cell-mediated protection as well (46). The importance of B cells in this line of immune defense may relate to their property to present Ags to T cells. It remained unclear how Ag presentation was achieved since processing and presentation of Ags by naive B cells was not observed. In this study, we provide a missing link in these observations by showing that the IgM^+ B cells can internalize viable bacteria and very efficiently induce Th activation. However, IgM secretion can also be induced by BCR-mediated *Salmonella* uptake and activation alone, albeit less efficient than observed with additional $CD4^+$ T cell help. The rapid secretion of IgM before B cells encounter $CD4^+$ T cells represents a first line of specific immune responses to pathogens and may represent the remaining humoral response when $CD4^+$ T cell help fails, as is the case in HIV patients. In conclusion, we demonstrate for the first time that bacterial uptake via the BCR by B

cells forms a highly efficient pathway to generate an immediate antimicrobial humoral immune response.

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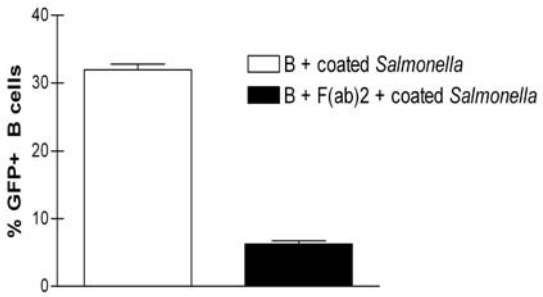
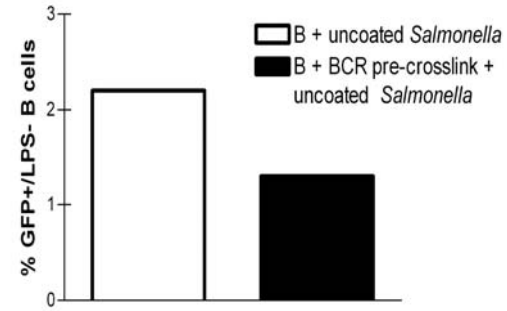


Supplemental figure S1. Blocking of the BCR with $F(ab)_2$ fragments.
Primary B cells were pre-incubated with $F(ab)_2$ fragments of the anti-IgM antibody before incubation with anti-IgM coated *Salmonellae*. Data are from five different donors, error bars respresent SEM.



Supplemental figure S2. BCR internalization before incubation with bacteria.
Primary B cells were pre-incubated with an anti-IgM antibody followed by incubation with a goat-anti-mouse antibody to achieve partial BCR internalization before incubation with uncoated *Salmonellae*. Data are one representative set of experiments from four different donors.

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